PATENT
0512-1352

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of

Christophe D'HULST, et al.	Conf. 9478
Application No. 10/594,526	Group 1638
Filed November 27, 2006	Examiner Brent Page

METHOD FOR IMPROVING PLANTS

DECLARATION UNDER RULE 132

Assistant Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Christophe D'HULST, hereby declare as follows:

I am one of the inventors of the above-identified U.S. patent application. My relevant background and experience are set forth on the attached CV.

I have read the Official Action mailed January 22, 2009, and I am familiar with the present application. In reviewing the Official Action, there does not appear to be any appreciation for the fact that the phenotype observed in the At-PHO1- line is linked to the only deficiency in the plastidial starch phosphorylase activity.

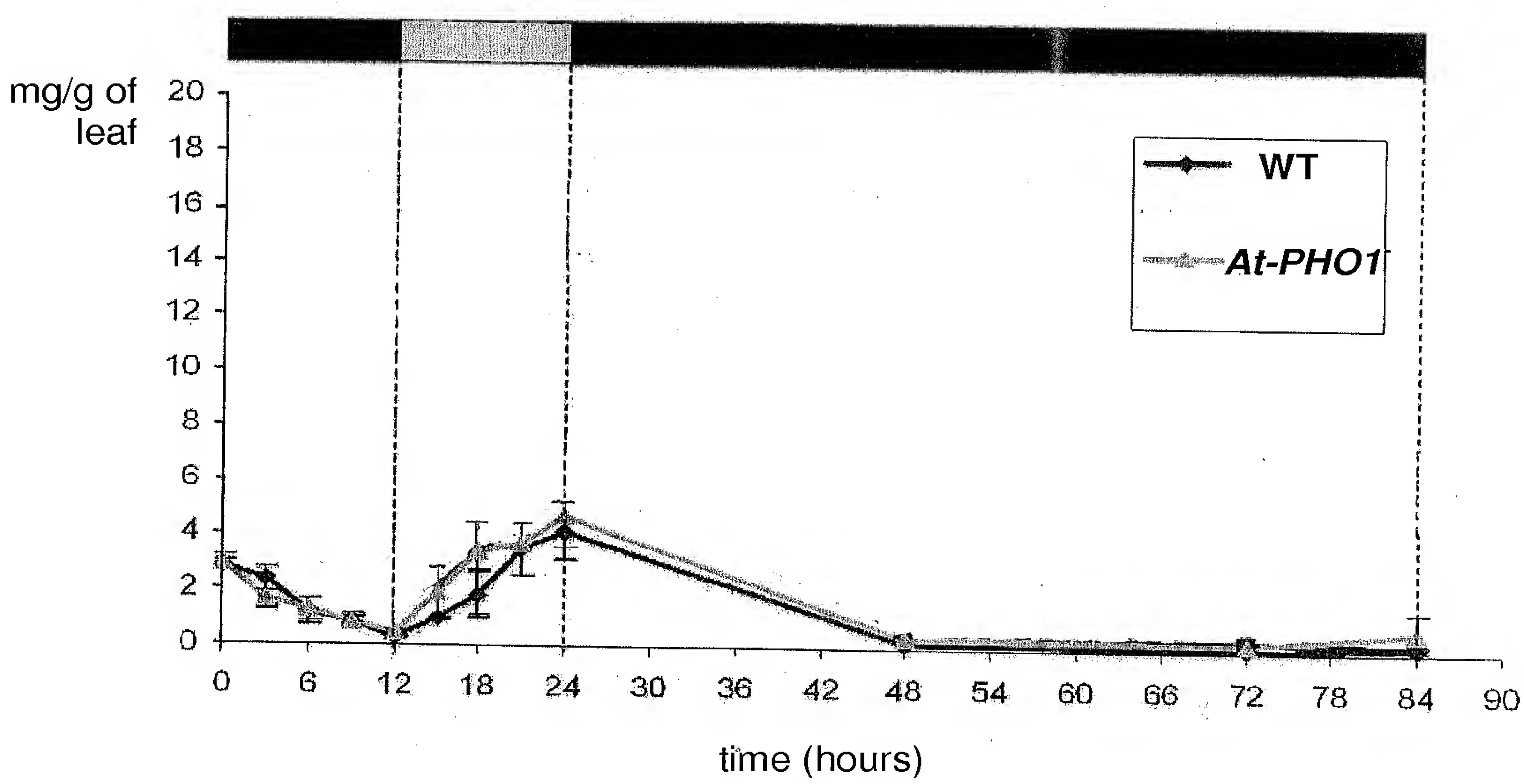
To demonstrate this fact, I have performed the following study which is a complete phenotypic study of the *Arabidopsis* line

At-PHO1$^-$ corresponding to the DDS72 line described in the application.

The rate of starch synthesis and degradation was determined in the *At-PHO1*$^-$ line and a wild-type line by performing a day/night cycle in phytotron, wherein the day and the night each last 12 hours. The day period was then followed by an extended period of night of 60 hours. During this cycle, starch was extracted and titrated from each line every three hours during the 12 hour-periods and every 24 and 12 hours during the extended period of night.

It was observed the mutant *At-PHO1*$^-$ line accumulated more starch than the wild-type line at the end of the day for the same fresh weight (Figure 1).

Figure 1: Accumulation of starch in wild-type lines (WT) and *At-PHO1*$^-$ *lines*. The amounts of starch are expressed in mg/g of fresh leaf. The grey rectangle represents the period of light (12 hours) and the black rectangle represents the period of night (12 hours).



Soluble glucans and polysaccharides were also assayed (Table 1). The *At-PHO1⁻* mutant did not show any notable modification compared to the wild-type (WT).

Table 1: Amount of soluble glucans and polysaccharides accumulated in leaves of different lines. The amounts are expressed in mg/g of leaf and the results represent the mean of 5 assays for each line. The plants were cultured in phytotron with a 12 hour- day period and a 12 hour- night period. The extraction was made on the plants at the end of the day.

	WSG	**Free Glc**	**WSP**
WT	0.18	0.17	0.01
At-PHO1⁻	0.24	0.25	0.03

WSG: water soluble glucans; WSP: water soluble polysaccharides; Glc: glucose. WSP = WSG - free Glc.

A kinetics of growth was also carried out in 12 hour-day / 12 hour-night growth conditions in phytotron. The *At-PHO1*$^{-}$ displayed an almost normal growth (Figure 2), but the weight of the above ground organs was slightly higher than the wild-type one (Figure 3).

Figure 2: Growth comparison. Pictures of the wild-type line and of the *At-PHO1*⁻ line were regularly taken after the seeds germination. The pictures are shown at the same scale to allow a direct comparison between the different lines. The numbers on the left indicate the number of days after germination. The plants were cultured in phytotron with a 12 hour-day period and a 12 hour-night period.

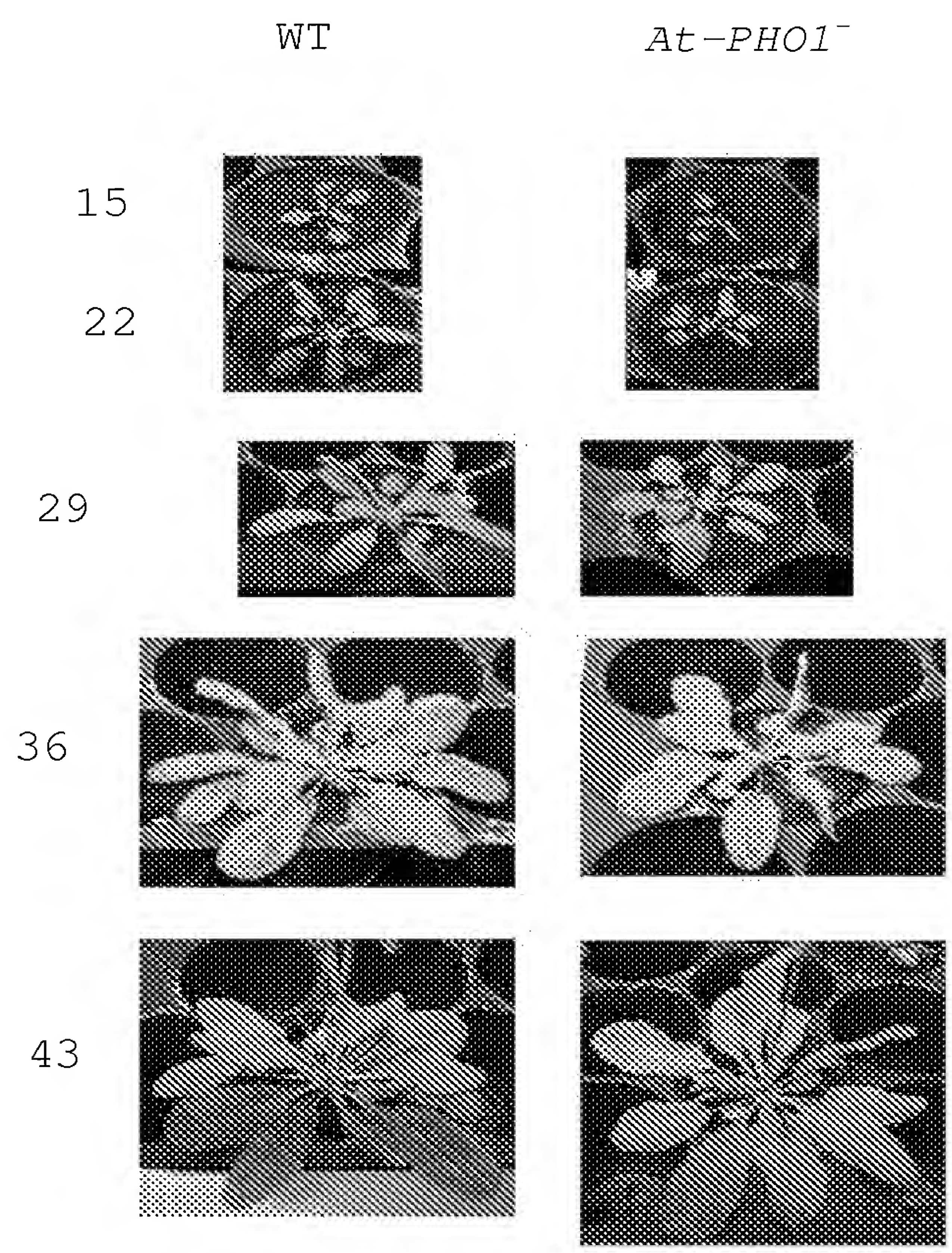
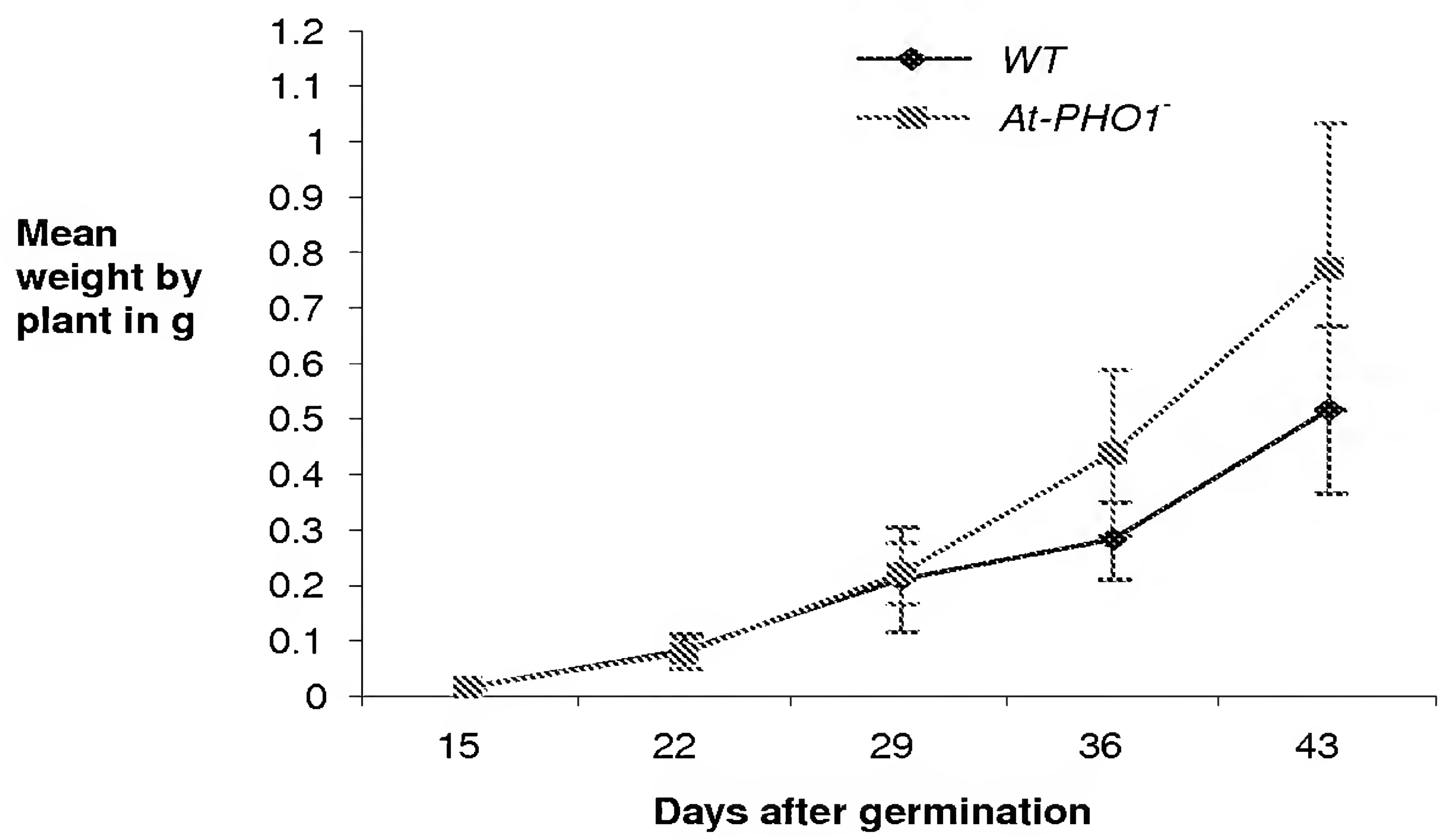


Figure 3: Comparison of weight of the above ground organs of different lines. The above ground organs were collected 15, 22, 29, 36 and 43 days after germination and immediately weighted. The plants were cultured in phytotron with a 12 hour-day / 12 hour-night period.



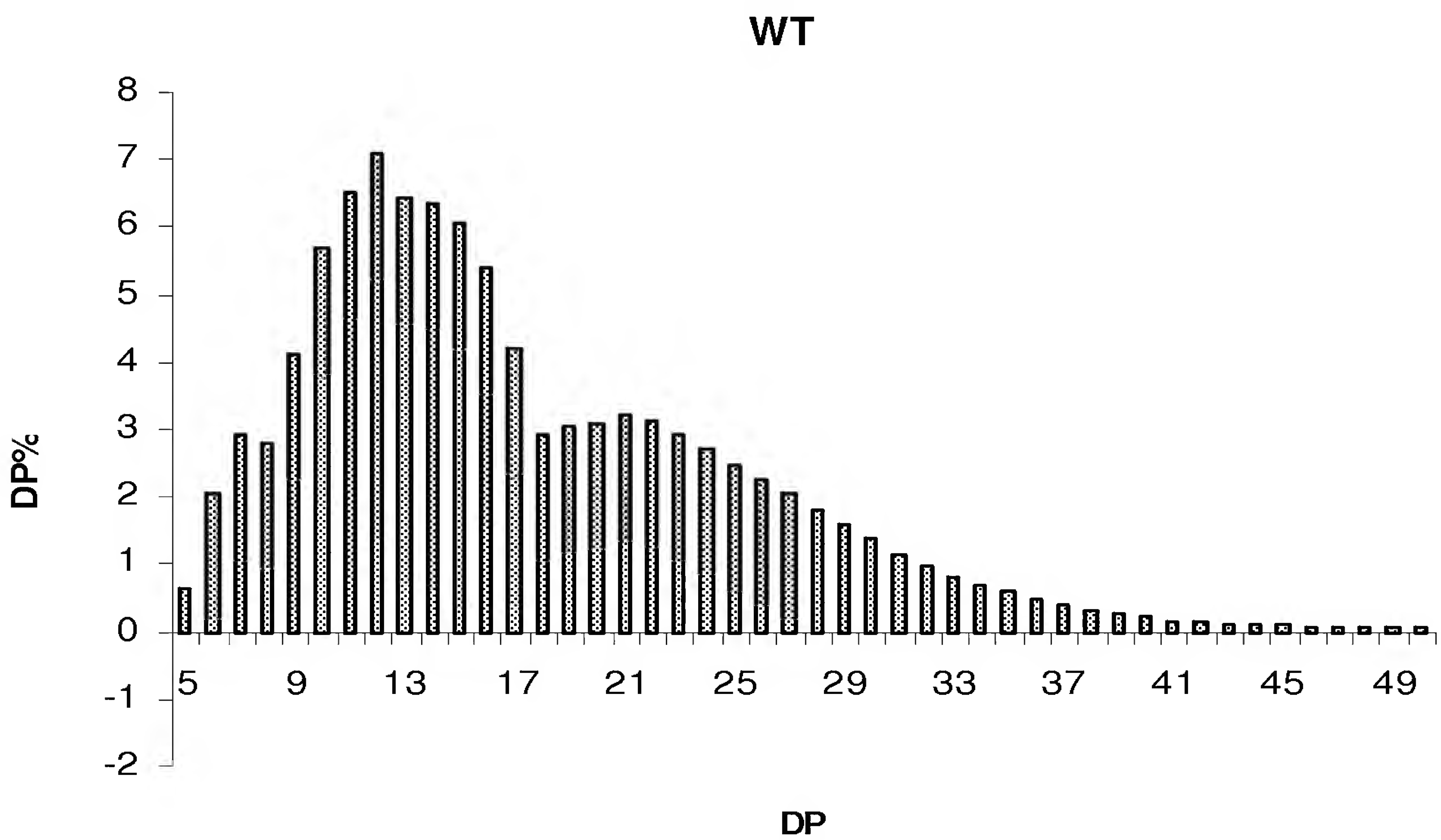
The structural analysis of the starch of the mutant line was then performed. A size exclusion chromatography was first used. This analysis enabled to observe that the iodine-amylopectin complex wave length value at the maximum of absorption (λmax) was essentially identical in the wild-type and the *AtPHO1*$^{-}$ lines (Table 2). Moreover, the mutant also contains the same amount of amylose as the wild-type.

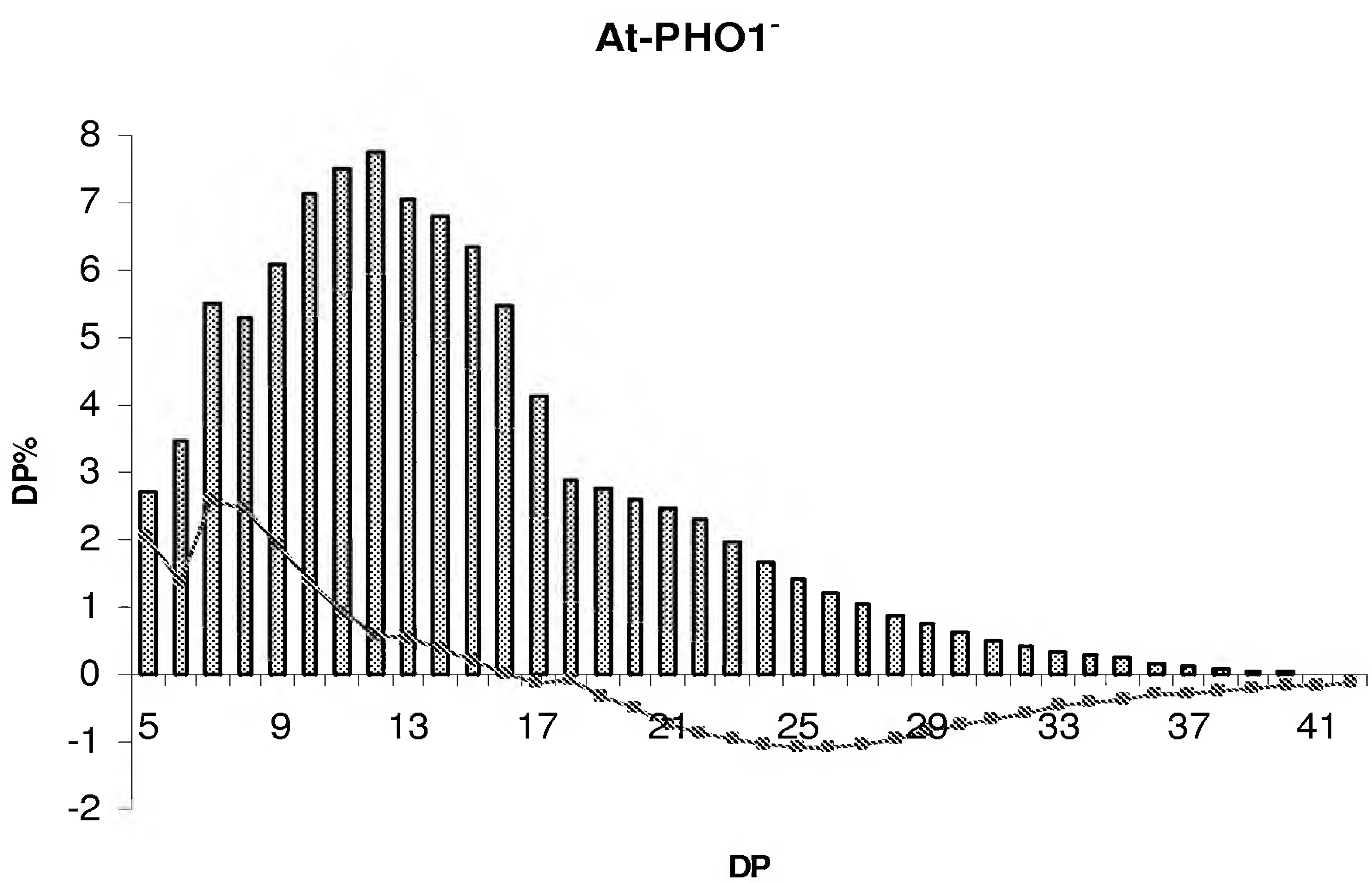
Table 2: λmax of amylopectin and amylose rate (% amylose) in wild-type and *AtPHO1⁻* lines. These analyses were carried out after size exclusion chromatography of total starch on a CL-2B sepharose column.

	λmax of amylopectin (nm)	**% amylose (standard deviation)**
WT	566 (n=4)	17 (4.2)
At-PHO1⁻	563 (n=5)	20 (7.4)

In order to highlight the structural difference, the length distribution of the glucans constitutive of the amylopectin fraction was obtained by capillary electrophoresis after enzymatic debranching of the polymer (Figure 4). This analysis showed an increase in the amount of short glucans (DP 5 to 12) in the amylopectin of the *At-PHO1⁻* mutant. A decrease in the amount of longer glucans DP 20 to 40 was also observed in this line. Thus the starch phosphorylase is necessary for the synthesis of normal starch in the *Arabidopsis thaliana* leaves.

Figure 4: Comparison of the length distribution of the glucans constitutive of amylopectin in different lines. The histograms represent the glucans length distribution and the red curve represents the difference plot between the mutant and the wild-type lines. The ordinate axes correspond to relative percentages of each chain for the histograms or the relative difference (in %) between the two amylopectins for the curve. The abscissa axes correspond to the different polymerization degrees (DP) of the glucans.





The activities of other enzymes of starch metabolism were also studied.

Different hydrolytic and phosphorolytic activities involved in starch metabolism were assayed in vitro. The results of these assays did not show any significant difference between the different lines for the assayed activities (Table 3).

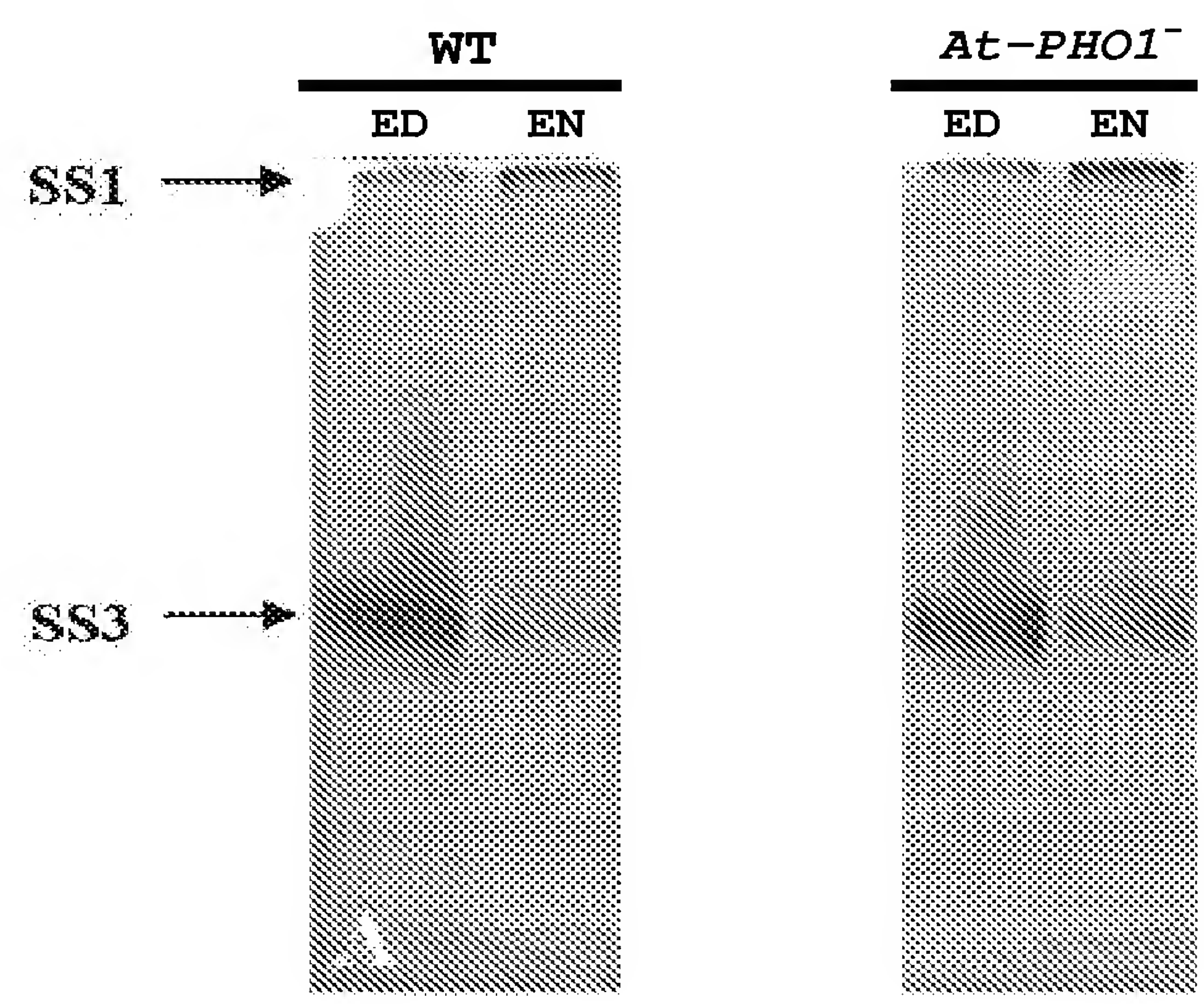
Table 3: Assays of hydrolytic and phosphorolytic activities in the wild-type and *At-PHO1*⁻ lines. The α-amylase activity is expressed in µmoles of paranitrophenol/min/mg of protein. The D-enzyme activity and maltase activity are expressed in mmoles of glucose/h/mg of protein. The phosphorylase activity is expressed in mmoles of G1P/min/mg of protein. The pullulanase activity is expressed in nmoles of maltotriose/min/mg of protein. Plants were cultured in phytotron for a 12 hour-day and 12 hour-night period. Extractions were performed at the end of the day (ED) and at the end of the night (EN).

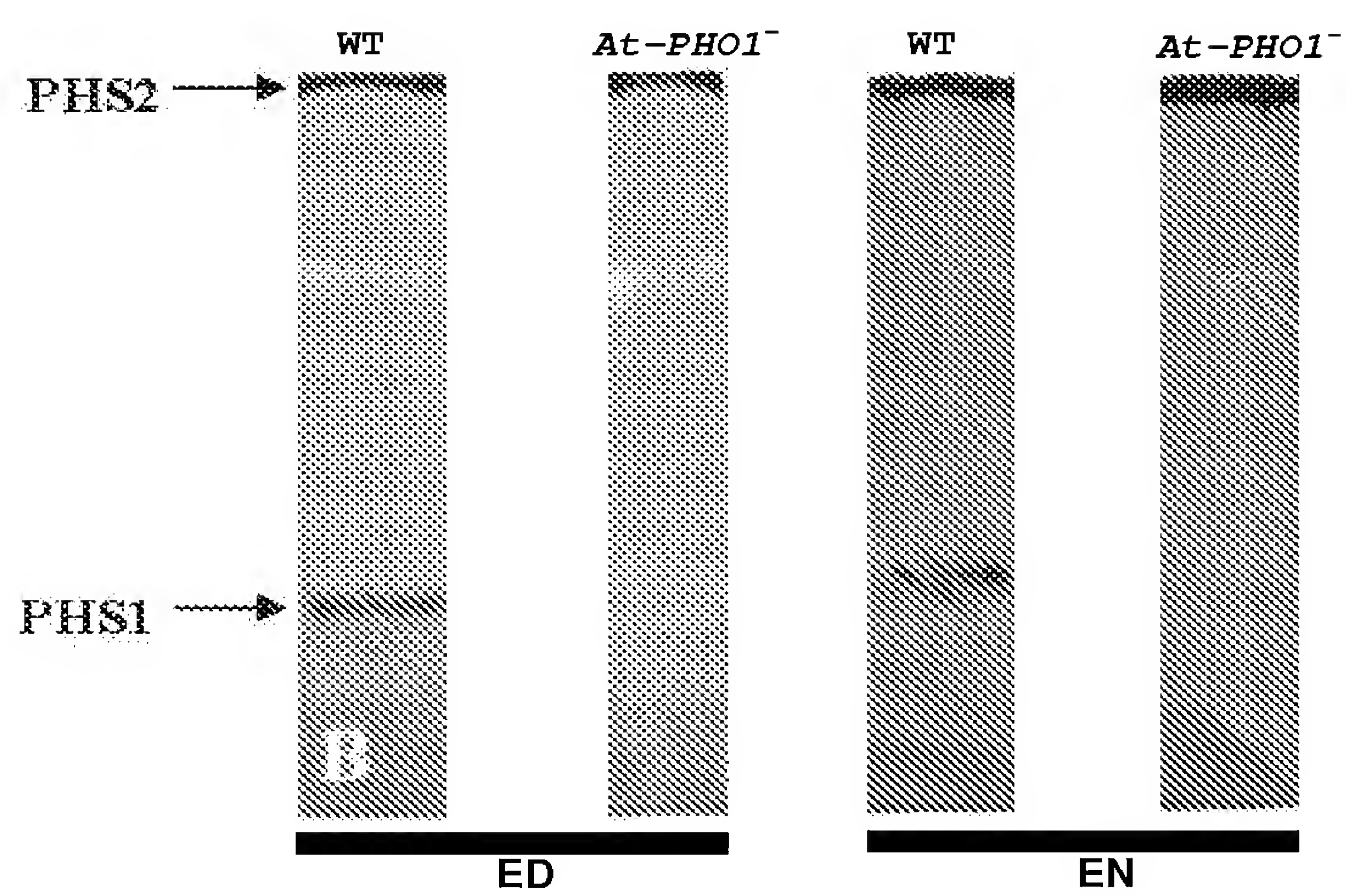
	α-amylase	D-enzyme	phosphorylase	pullulanase	maltase
WT ED (n=1)	0.23	4.14	628	48,3	1.02
WT EN (n=2)	0.14	3.26	772	30,5	0.96
***At-PHO1*⁻ ED (n=1)**	0.13	3.38	5	28,4	1.39
***At-PHO1*⁻EN (n=2)**	0.14	2.52	244	24,8	0.99

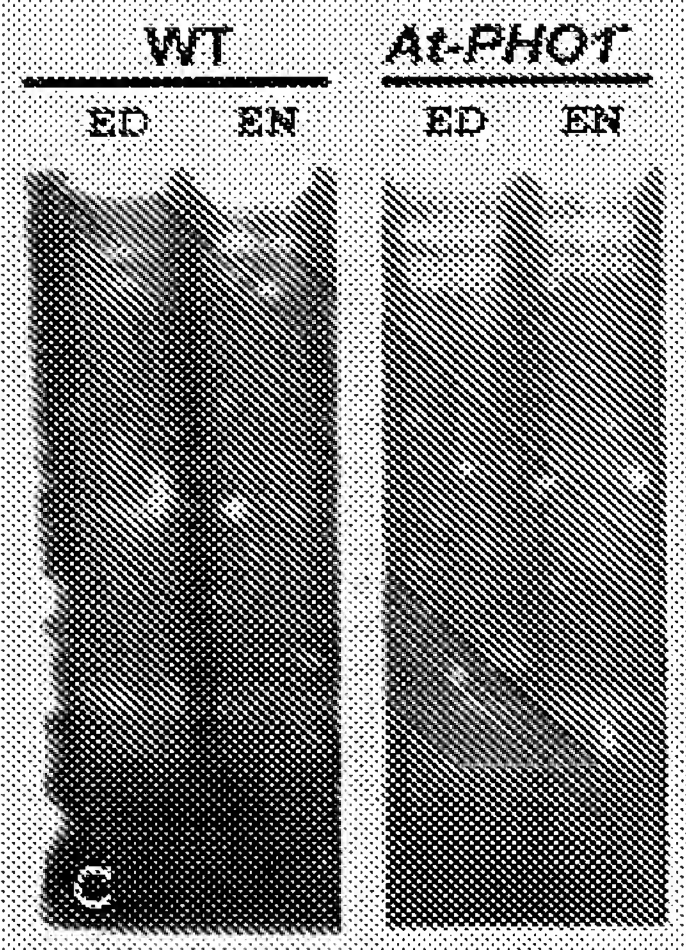
Gels of enzymatic activities (zymograms) were also realized to study the different enzymatic activities complementary to those described in Table 3. The analyses of starch synthases (Figure 5A) and starch modifying hydrolytic enzymes (Figure 5C) thus did not show any difference between wild-type and mutant lines. On the contrary, the analysis of α-glucan phosphorylases showed that the plastidial starch phosphorylase was absent in the *At-PHO1*⁻ line

(Figure 5B).

Figure 5: Gels of enzymatic activities visualizing the soluble starch synthases, α-glucans phosphorylase and starch modifying activities. (A) Gel showing the soluble starch synthase activities SS1 and SS3. **(B)** Gel showing the α-glucan phosphorylases activities PHS1 and PHS2. **(C)** Gel showing the starch modifying activities. Gels in (A) and (B) contain rabbit liver glycogen. Gels in (C) contain potato soluble starch. ED: protein extraction at the end of the day. EN: protein extraction at the end of the night.







These different analyses confirm that the phenotype observed in the At-*PHO1⁻* line is only linked to the deficiency in the plastidial starch phosphorylase activity.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Christophe D'HULST

Date July 21st, 2009